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A technique to decontaminate soil microarthropods for introduction to gnotobiotic systems*

John C. Moore[1, 2, 3], John A. Trofymow[4] and Carole R. Morley[1, 2]

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1. Introduction

Introducing soil microarthropods to gnotobiotic systems can be an effective way to study their interaction with other organisms and role as decomposers. Although a number of studies have addressed trophic relationships in soil (Hanlon & Anderson 1980; Anderson *et al.* 1981; Wallwork 1983), quantifying soil arthropod contributions to these relations has been hindered by fungal and bacterial contamination. High microarthropod mortality and changes in fecundity have been a common occurrence in microcosm studies dealing with arthropod-microbial interactions. This has been generally a result of microbial overgrowth in the microcosms (Moore unpublished data; Usher *et al.* 1982) as the arthropod exoskeleton and gut are replete with fungal spores and bacteria. The ornamented cuticles of microarthropods, e.g., Collembola (Lawrence & Massoud 1973) serve as microsites for bacterial growth and attachment for fungal spores and hyphae. Therefore, addition of arthropods to gnotobiotic systems may also introduce significant numbers of unknown bacterial and fungal contaminants. In many instances, introduced contaminants are an uncontrolled variable that may contribute to measured parameters, particularly in decomposition studies.

Attempts have been made to minimize contamination. Snider (1973) suggested frequent scraping of plaster-charcoal substrates used to rear Collembola with periodic transfer to new plates. For rearing Collembola, Goto (1960) successfully controlled fungal growth with Nipagin-M (methyl-p-hydroxybenzoate). Hanlon (1981) used Crystamycin to inhibit bacterial growth in sand when studying Collembola grazing on fungal colonies. With the exception of Snider (1973), the above methods have treated substrates with biocides. It is our opinion that enhanced degradation of these chemicals by microbial populations and undesirable non-target effects may occur in microcosm studies. Decontamination of microarthropods prior to addition to gnotobiotic microcosm systems rather than applying biocide to substrate would eliminate these concerns.

For successful decontamination the following prerequisites must be met: the biocides must be effective against a broad spectrum of microbes and cannot kill, reduce fecundity and fertility, alter egg/arthropod development time; or change behavior. The methods presented here have efficiently decontaminated fungivorous and nematophagous soil arthropods with no adverse bionomic effects on their life histories. Results of decontamination trials and sterility checks using a fungivouros collembolan *Folsomia candida* (Willem) [Collembola: Isotomidae] and nematophagous mite *Hypoaspis sp.* [Acari: Laelapidae] are presented to demonstrate the method's effectiveness.

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[1]Department of Zoology and Entomology, Colorado State University, Fort Collins, CO 80523.

[2]Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523. [3] Department of Statistics, Colorado State University, Fort Collins, CO 80523. [4] Environment Canada, Pacific Forest Research Centre, 506 West Burnside Road, Victoria, B.C. V8Z 1M5 Canada.

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2. Materials and methods

2.1. Decontamination procedure

The decontamination procedure involves two stages: Stage I was devised to eliminate external fungi and bacteria, while stage II was designed to purge gut microflora.

Stage I: A solution of 70% sterile water, 20% Fungizone (lyophilized; Amphotencin B-250 µg/ml), and 10% (10 µg/ml) streptomycin sulfate (referred to as FSS) was used as an exoskeleton decontaminant. Five ml of FSS were pipetted into a 30×15 mm sterile petri dish and arthropods were added. The duration of stage I depended on whether the animals sank or floated on FSS. If animals sank, a minimum of 5 h of exposure were allowed. For animals that floated, 1 to 2 d were allowed to ensure that all parts came in contact with FSS. After adequate exposure, arthropods were transferred to stage II.

Stage II: To eliminate microflora from the gut, arthropods were fed a food source treated with FSS. Treated food passed through the gut and could kill or prevent growth of bacteria and fungal spores within. Different methods were developed to decontaminate fungivorous and nematophagous forms. Fungivorous forms, Collembola in particular, were found to ingest nutrient agar (MOORE, unpublished data). Therefore a mixture of 8 g nutrient agar per liter of FSS (FSA) was used to purge *F. candida* gut flora. *Hypoaspis sp.* were placed on FSA and fed live nematodes that had been emersed in FSS for 1 week.

All equipment and chemicals used were sterile and operations were conducted under a laminar flow hood to prevent aerial contamination. To avoid denaturing, biocides were added to autoclaved (20 min at 21 psi) water cooled to room temperature (20 °C) or in the case of FSA, agar was allowed to cool but not solidify (40 °C) before biocides were added. Stages I and II were conducted sequentially. A sequence or series of sequences always ended with an additional stage I to minimize recontamination and facilitate transfer to gnotobiotic systems.

2.2. Methodological experiments

Two experiments were conducted to test the method's effectiveness. Fungivorous *F. candida* Collembola: Isotomidae) and nematophagous *Hypoaspis sp.* (Acari: Laelapidae) were used as test animals. Both species were obtained from laboratory cultures.

Forty *F. candida* (Trial I) underwent two decontamination sequences (i.e., Stage I + Stage II + Stage I + Stage II + Stage I). These *F. candida* were referred to as the treatment group. An equal-sized control group had sterile water and nutrient agar substituted for FSS and FSA, respectively. After two decontamination sequences, 10 treatment and five control *F. candida* were selected at random and individually reared in 20×60 mm petri dishes on nutrient agar. The plates were incubated at 20 °C for 3 weeks. Cultures were inspected daily for bacterial and fungal contamination, mortality, molts, and egg production. The number of *F. candida* that molted and the number of days from placement on nutrient agar until an animal's first molt were used as population molt frequency and instar duration indices. After 3 weeks, all uncontaminated treatment *F. candida* and two randomly selected controls were squashed and transferred to nutrient broth. The broth was incubated at 25 °C for 1 week and checked for microbial growth. Nutrient agar plates containing eggs were incubated for an additional 3 weeks and the number of were counted at the end of this period. The procedure was repeated using 91 control and 138 treatment animals (Trial II). Similarly 5 control and 10 treatment animals were selected for sterility checks, molting and egg production. Due to reports of contamination in our Laminar flow hood area by other investigators, an additional 5 nutrient agar petri dishes (hood control check) were opened and closed in the Laminar flow hood area and incubated as above to check for contamination areally introduced.

Effects of FSS/FSA on *F. candida* fertility and survivability in gnotobiotic microcosms were also determined. Five groups of control and treatment *F. candida* (~ 20 animals/group) underwent two control/treatment decontamination sequences. From each group 2 animals were selected randomly and placed in 250 ml Erlenmeyer flasks containing 5 g of sterile soil (autoclaved 3 h), 5 ml of sterile water, and 5 g of autoclaved brewers yeast (20 min). A sterile foam plug was inserted into the flask opening and the flasks were placed in $^1/_2$ litre screw-cap jars. The animals were incubated in the flask/jars for 5 weeks at 23 °C. After one week, surviving adults were counted. Approximately every 2 days the jars were opened to allow volatiles to escape, replenish the air supply, and record presence and absence of juveniles. At the end of 5 weeks, the juveniles in each Erlenmeyer microcosm were counted.

Hypoaspis sp. underwent three decontamination sequences with stage I similar to *F. candida;* however, fewer individuals were used. In stage II, bacterial feeding nematodes *(Acrobeloides sp.)* were used as prey. *Acrobeloides sp.* had been cultured on agar plates containing a reference soil solution (RSS), 2% agar and glucose, after HERZBERG *et al.* (1978) and inoculated with a single bacterial species *(Pseudomonas paucimobilis).* The *Acrobeloides sp.* were immersed in FSS for 1 wk before being fed to treatment mites. Control *Hypoaspis sp.* had sterile water and nutrient agar with *Acrobeloides sp.* that had been immersed in sterile water for 1 wk prior to addition to FSA as substitutes for stages I and II, respectively. Efficacy of sterilization was tested by selecting 5 treatment and 5 control *Hypoaspis sp.* at random and rearing them individually, as described for *F. candida.* Treat-

ment and control *Hypoaspis sp.* were incubated at 20 °C for 2 d, then squashed and transferred to nutrient broth. Nutrient agar dishes and broth were incubated for 3 weeks at 25 °C and monitored for contamination.

2.3. Statistical analysis

Differences between treatment and control group decontamination and molt counts were determined for each trial by Fisher's exact test for small samples (McGuire *et al.* 1967) and combined after Rao (1973). All other variables collected on sterility check trials I and II were analysed using a randomized block design, blocked on trials. The ANOVA was performed on $\text{Log}_e (X + 1)$ transformed data.

The fertility/survivability check, conducted in Erlenmeyer microcosms, was analysed in two ways; one week survivability was compared by Pearson's chi-squared test for equal proportions; and juvenile production was compared with the Mann-Whitney analysis.

3. Results and discussion

On day four of trial I and days 2 and 3 of trial II, one treatment *F. candida* escaped from the petri dish. Because controls showed bacterial growth and egg production and molts within two days after placement on nutrient agar, these replicates were included in molt and egg comparisons and monitored for contamination.

The procedure was effective in decontamination (Table 1). After three weeks, all control *F. candida* and only 1 treatment from trial 1 and 2 from trial II showed contamination. Similarly, *Hypoaspis* yielded only a single contaminated treatment plate after 3 weeks. The nutrient broth sterility test found 2 additional contaminated treatment *F. candida* in trial II. The hood control nutrient broth sterility check indicated that the Laminar flow hood had introduced a fungal contaminant identified as a *Penicillium sp.* Two of the four contaminated treatment animals but none of the control, had this same fungus in their plates as the sole contaminant. The data presented in Table 1 included analysis with and without adjusting for the hood contamination.

The procedure did not adversely affect *F. candida* life history. Population molt frequency did not differ between treated and untreated animals (Table 2). Egg production was limited for both treatment and control *F. candida* (Table 3 A): no significant difference in egg production by *F. candida* in both treatment and control groups was found. Fertility in gnotobiotic systems was higher among treated than untreated *F. candida* (Table 3 B).

Table 1. Number of animals *(n)* decontaminated. Based on three weeks incubation (20 °C) on nutrient agar followed by one week incubation (25 °C) in nutrient broth.

Variable	Control	Treatment	P value
F. candida			
Trial I	0 (5)	9 (10)	P = .002
Trial II	0 (5)	6 (10)	P = .042
Trial II*	0 (5)	8 (10)	P = .007
Trial I + II*	0 (5)	17 (20)	P ≤ .001
Hypoaspis sp.	0 (5)	4 (5)	P = .024

* Less the Laminar flow hood introduced contamination.

Table 2. Number of *Folsomia candida* that molted during the three week nutrient agar incubation and mean days to the first molt after placement on nutrient agar

	N	No. animals molted	Mean days to molt
Control	10	9^{A*}	4.78^{A}
Treatment	20	12^{A}	4.58^{A}

* Similar letters indicate no significant differences, P ≥ .05.

Table 3 A. *Folsomia candida* egg production and fertility on nutrient agar

	N	No. reproducing adults (layed eggs)	No. eggs/ reproducing adult	Days to egg	No. juveniles/ reproducing adult
Control	10	3	20.67^{A*}	7.0^{A}	0^{A}
Treatment	20	7	14.71^{A}	6.71^{A}	2.57^{A}

*Similar letters indicate no significant differences, $P \geqq .05$.

Table 3 B. *Folsomia candida* fertility/mortality results from Erlenmeyer microcosms

	N	1 wk mortality	Juveniles	Juveniles/microcosm (after 5 weeks)
Control	28	19^{A*}	52^{A}	3.71^{A}
Treatment	28	5^{B}	671^{B}	43.93^{B}

*Similar letters indicate no significant differences, $P \geq .05$.

Table 4 A. Survival results after 2 decontamination sequences

		N	Alive	
Folsomia candida				
Trial I	control	40	12	$P > .05$ (N.S.)*
	FSS/FSA	40	11	
Trial II	control	91	64	$P > .05$ (N.S.)
	FSS/FSA	138	113	
Hypoaspis sp.				
	control	6	5	$P > .05$ (N.S.)
	FSS/FSA	6	5	

*Not significant
Note: Animals that escaped were scored as dead.

Table 4 B. *Folsomia candida* one week survivability results in Erlenmeyer microcosms*

	Number of microcosms	Number of microcosms with X_i animals ($X_i = 0, 1, 2$)			
		0	1	2	
Control	14	7	5	2	obs $X^2 = 10.69$
FSS/FSA	14	0	6	8	$P \leq .005$

* Two animals/Erlenmeyer

No increased mortality was noted as a result of fungizone and/or streptomycin sulfate (Table 4 A). The majority of deaths occurred during stage I, probably due to immersion in the water/FSS. Deaths may be minimized by using new petri dishes with a static charge and not agitating the dish after arthropods were added. Significant losses among *F. candida* also occurred during transfer from FSS to FSA. These Collembola are furculate and difficult to control during transfer. Initially, FSS dishes were tilted and *F. candida* not trapped by the solution's surface tension accumulated at the lower edge and jumped onto FSA. Live *F. candida* trapped by surface tension were transferred with a sterile loop to a sterile water bath to remove FSS from the loop and then placed on FSA. *Hypoaspis sp.* were easily transferred with a sterile loop and none were lost. Survival was higher in gnotobiotic microcosms among treatment than untreated *F. candida* (Table 4 B).

Clearly, this method was effective in bacterial and fungal decontamination. Nutrient agar plates and broth were incubated for sufficient time to yield contaminants. More im-

portant, all sources of contamination were addressed. *Folsomia candida* eggs, feces, and body fluids yielded no contamination in all but five treated animals. *Hypoaspis sp.* yielded only one contaminant when feces, exoskeleton, and body fluids were tested. Furthermore, no difference in mortality, egg production or instar duration occurred as a result of the procedure. The method has broad application. Aside from increasing microarthropod survivability and/or maintaining sterility in gnotobiotic systems, studies on food quality and digestibility in the absence of microflora and feeding preference studies under gnotobiotic condition can be conducted. Adjustments in FSS mixtures and duration of FSS/FSA are recommended to suit specific needs. In studies where microarthropod survival would be more desirable than complete gnotobiotics, shorter and fewer decontamination sequences are necessary. For example, in the fertility studies on *F. candida*, between 20—60 % of the treated systems could be expected to be contaminated based on the 60—90 % efficiency figures, yet only 1 of 14 treated systems failed to produce young compared to 12 of 14 untreated systems. Finally, adjustments in chemical concentrations of FSS/FSA and formulation (other biocides) may be necessary depending on the microflora present. Currently, further tests are being conducted on more arthropod species and other soil invertebrates.

4. Zusammenfassung

Eine Dekontaminations-Technik zur Einbringung von Boden-Mikroarthropoden in gnotobiotische Systeme

Für gnotobiotische Studien wurde eine Zweistufen-Laboratoriums-Prozedur zur Dekontamination von Collembola und Acari entwickelt. In der Stufe I wurde versucht, die Tiere durch Flotation und Eintauchen in eine Fungizone-Streptomycin-Lösung (FSS) von intestinalen Kontaminanten zu befreien. Die [Wirkung der] Prozedur wurde mit Versuchstieren zweier Arten, die fungivore Collembole *Folsomia candida* (Willem) und die nematophage Milbe *Hypoaspis sp.* [Acari: Laelapidae] geprüft. Für beide Arten wurde eine signifikante Reduktion der Kontamination und kein Anstieg der Mortalität ermittelt. Die bionomischen Daten für *Folsomia candida* zeigten keine Zunahme der Entwicklungsdauer oder Abnahme der Eiproduktion. Fertilitätsstudien unter sterilen Kautelen ergaben eine geringere Mortalität und höhere Fertilität der behandelten (dekontaminierten) Versuchstierpopulationen von *Folsomia candida* im Vergleich zu unbehandelten Vergleichstieren.

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Corresponding author: Dr. J. C. Moore (Address see page 185)

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A two-stage laboratory procedure was developed to decontaminate Collembola and Acari for use in gnotobiotic studies. In stage I, animals are floated/immersed on/in a Fungizone-Streptomycin Solution (FSS) to remove external contaminants. In stage II, animals are fed FSS treated food to remove internal contaminants. The procedure was tested on two species, the fungivorous collembolan *Folsomia candida* (Willem) [Collembola: Isotomidae] and nematophagous mite *Hypoaspis sp.* [Acari: Laelapidae]. For both species there was a significant reduction in contamination and no significant increase in mortality. Life history data on *F. candida* indicated no increase in instar duration or decrease in egg production. Fertility studies in sterile microcosms found lower mortality and higher fertility among the treated (decontaminated) *F. candida* than untreated controls.
Key words: Microarthropods, Collembola, Acarina, decontamination, gnotobiotic microcosms.